

Plague into the 21st Century

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As an ancient scourge, plague caused deadly epidemics in medieval Europe, and in the 20th century, it caused extensive mortality in India and Vietnam. Crossing into the 21st century, it has attracted particular attention as a potential bioweapon, for which a new vaccine needs to be developed. Human plague syndromes are mainly bubonic, septicemic, and pneumonic, all caused by the bacterium *Yersinia pestis*. Considerable strides have been made in understanding the causative organism's virulence, although plague has persisted as a killer disease in Africa, Asia, and the Americas [1]. This update focuses on epidemiological trends, bacterial virulence, diagnosis, and treatment of plague.

EPIDEMIOLOGY

Occurrence. Plague is enzootic in rodents, with infection sometimes reaching humans, usually by flea bites, on the continents of Africa, Asia, South America, and North America. Most cases are bubonic plague, so-called because patients have fever and a bubo, which is a swollen, tender, necrotic lymph node often in the femoral, inguinal, or axillary region and teeming with *Y. pestis*. Other cases are septicemic, in which bacteria circulate in the blood but do not localize in a bubo, and some are pneumonic, with cough and pulmonary infiltrates. For the decade 1994–2003, the World Health Organization reports that the number of confirmed and suspected human cases of plague in all countries was 28,530, with 2015 deaths, for a case-fatality rate of 7.1% (Table 1). In 1994, India reported 876 cases of plague,

but conflicting reports have been published regarding whether these cases were truly plague [5–8]. Evidence that these cases were not plague included lack of secondary cases among patients in close contact with pneumonic patients in Surat, lack of bubonic plague cases in Surat, a low mortality rate of 6.2% (54 of 876 cases), lack of dying off of rodents, lack of bacterial isolations in a microbiology laboratory during the epidemic, and lack of virulence after animal inoculation using 11 isolates obtained from contaminated cultures after the epidemic subsided, despite that plague genes were found in them by polymerase chain reaction (PCR). In Algeria, plague reemerged in 2003, with 18 reported cases and 1 death of a patient, after the country had been free of disease for 50 years [9]. The United States reported 61 cases of plague in 1994–2003. In 2006 in the United States, 13 cases, with 2 deaths, were reported in the states of New Mexico, Colorado, California, and Texas [4].

Transmission and animal reservoirs. Humans acquire this zoonotic infection when animal fleas aberrantly bite them, sometimes prompted by an animal's death from plague, after which the flea seeks a new source of blood meals. The incubation period from fleabite to symptomatic disease is 2–10 days [10]. Humans can be viewed as playing no role in the maintenance of plague in nature because rodent populations and their fleas suffice and because humans are poor transmitters of short-lived outbreaks of pneumonic plague. Most infected fleas come from the domestic black rat *Rattus rattus* or the brown sewer rat *Rattus norvegicus*. The most common and efficient flea vector is *Xenopsylla cheopis*, but many other flea species can transmit plague. The oriental rat flea *X. cheopis* is more susceptible than are other fleas to having the proventriculus of its digestive tract blocked by a blood meal containing *Y. pestis*, because bacterial growth enables formation of an aggregative biofilm on the spicules of

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Table 1. Cases of Human Plague in 1994–2003 in the Countries That Reported >100 Confirmed or Suspected Cases

Country	No. of cases	Case-fatality rate, %
Madagascar	12,270 ^a	8
Congo	3619	10
Tanzania	3527	7
Mozambique	2387	1
Vietnam	1331	6
Malawi	900	2
India	892 ^b	7
Uganda	654	17
Peru	631	3
Zimbabwe	417	8
China	357	7

NOTE. Data are from [2].

^a Clinical forms from Madagascar in 1996–1998 reported as 97% bubonic and 3% pneumonic cases [3]. Other countries also reported predominantly bubonic plague. Septicemic plague was rarely reported, but in 2006, the United States reported that 5 (38%) of its 13 cases were septicemic [4].

^b Cases in India were predominantly pneumonic plague.

the proventriculus [11]. Blocked fleas are unable to clear their midguts of infected blood, leading them to bite repeatedly and to regurgitate bacteria into the skin of its next host. In Madagascar, flea transmission occurs mainly in *R. rattus* in the highlands, but in the coastal city of Mahajanga, house shrews also harbor infection [12]. In addition to flea transmission, some cases are caused by direct handling of animal tissues, when bacteria are inoculated through skin lacerations or when aerosolized bacteria are inhaled. Two of the cases in the United States in 2006 occurred in hunters who had skinned rabbits [4]. Animals associated with human cases that were found to be infected or to harbor infected fleas in the United States were white-tailed antelope squirrels, ground squirrels, rock squirrels, cottontail rabbits, jack rabbits, prairie dogs, deer mice, Colorado chipmunks, and wood rats [13, 14]. One case in India in 2002 occurred in a hunter who had killed and skinned a sick wildcat [15]. In Mongolia in 2002, there were 6 cases of plague, for which the most common mode of acquisition was among hunters who skinned sick marmots [2]. In Qinghai Province of China in 2004, there were 19 human cases, with 8 deaths; most of these cases occurred in people who had hunted and butchered marmots [16]. Domestic cats in the United States were the source of 23 cases of human plague from 1977 through 1998, caused by bites and scratches in most cases but caused by inhalation of cat respiratory secretions in 5 cases of primary pneumonia [10]. Cats become infected by eating rodents, but these carnivores play a minor role in plague epidemiology, probably because their fleas are inefficient transmitters of infection. Dogs and other canids are naturally resistant to plague.

A sick camel in Saudi Arabia in 1994 was the source of 5 cases, with 2 deaths; 4 persons who ate raw camel liver developed pharyngitis, and 1 person who had butchered the camel developed an axillary bubo [17].

Primary inhalational lung infection is a rare form of transmission but can propagate person-to-person outbreaks of plague. The index patient usually starts with bubonic or septicemic illness from a fleabite, which develops into secondary pneumonia from bacteremic spread. Coughing produces airborne droplets that are inhaled by family or other close personal contacts. In Madagascar in 1997, an outbreak occurred that affected 18 persons, with 8 deaths [18]. The index patient, who had bubonic plague with secondary pneumonia, spread infection to a traditional healer, who sucked bacteremic blood from the patient's skin, acquiring lung contagion that he passed to his family and another patient before he died. At a funeral for the healer, more cases occurred, because of airborne exposure. In the Himachal State of northern India in 2002, an outbreak of pneumonic plague resulted in 16 cases and 4 deaths [15]. This outbreak was started by a hunter who killed and skinned a sick wildcat, developing fever 5 days later, followed by cough and hemoptysis suggestive of pneumonia. Before he died, he infected his family and other patients in a hospital. In 2004, there were 4 cases of pneumonic plague reported in Uganda, with 3 deaths. Two of these cases were secondary pneumonia after bubonic and septicemic disease, and 2 cases were primary inhalational pneumonia in caregivers of the patients with secondary pneumonia [19]. Incubation periods for primary pneumonia are ~3 days after contact with a coughing patient, and death usually follows in another 3 days, unless an antibiotic is administered on the first day of symptoms or prophylactically. Outbreaks of pneumonic plague in recent years have been restricted to a small number of cases during a few weeks, because spread is inefficient by large droplets that require close contact with coughing patients in the last hours of terminal illness [20].

Susceptible persons. Patients of all ages and both sexes are susceptible to disease. Distributions by age and sex were not given in the World Health Organization report [2], but most cases in recent decades occurred in children, with a slight preponderance among male persons. In Madagascar in 1996–1998, 61% of confirmed and presumptive cases occurred in children and adolescents (age, 0–19 years) and 57% occurred in male persons [3]. In the United States in 2006, the age range was 13–79 years, and 8 of the 13 cases occurred in female persons [4]. Exposure of persons to infected fleas where local rodents are transmitting infection is most determinative. Occurrence of an epizootic with a visible dying off of rodents that harbored plentiful fleas increases the chance of human cases. High incidences of cases are associated with poverty, which results in substandard housing that is not rat proof. Warm climates in

Table 2. Virulence Factors of *Yersinia pestis* Encoded by Genes of Plasmids and Chromosomal Loci

Gene location, product(s)	Activities
Plasmid of 70 kb called the low-calcium response plasmid, pYV, or pCD1	
Type III secretion system	Insertion of Yops through a needlelike injectisome into neutrophils, macrophages, and dendritic cells [23]
V antigen	Anti-inflammatory activity through interleukin 10 secretion [24]; forms tip of type III secretion system [25]
Yops	Inhibition of phagocytosis, platelet aggregation, and cytokine production; deubiquitination of proteins and apoptosis of macrophages [26, 27]
Plasmid of 100 kb called pFra or pMT1	
Fraction 1 capsular antigen	Inhibition of phagocytosis [28]
Murine toxin	Survival of bacteria in flea gut [29]
Plasmid of 9.5 kb called pPst or pPCP1	
Plasminogen activator	Activation of host plasminogen to plasmin for lysis of clots; cleavage of extracellular matrix to promote bacterial spread [30]; bacterial multiplication in lung [31]
Chromosomal pigmentation locus (<i>pgm</i>)	
Hemin storage	Formation of obstructive biofilm in flea gut [11]
<i>Yersinia</i> bactin	Siderophore for iron transport [32]
Chromosomal locus under regulation of RovA	
pH 6 antigen	Inhibition of phagocytosis [33, 34]
Chromosomal locus for Ail proteins	
Outer membrane	Resistance to complement-mediated killing [35]
Chromosomal locus for cell wall synthesis	
Lipopolysaccharide that is rough due to lack of O antigen	Initiation of inflammatory responses leading to septic shock, also for anti-inflammatory nonstimulation of Toll-like receptor 4 [36]

NOTE. kb, kilobases; Yops, yersinial outer proteins.

developing countries give rise to exposure of skin to fleabites, because of persons' uncovered legs and feet. Unsettled conditions of war and relocations of refugees with lack of public health services favor plague because rodents will feed on garbage in greater proximity to people's dwellings.

Seasonality. Human plague in all countries of endemicity shows seasonal variation. The peak season corresponds to the timing of epizootics with dying off of susceptible rodents. These seasons often can be correlated with increases in fertility of rodent fleas, increases in rodent populations, and greater proximity of humans to infected animals. In the United States, the plague season is from February through August [13]. In Vietnam, cases occur mainly from January through April. The highlands of Madagascar have a peak season from October through February, but the coastal city of Maharanja experiences disease mainly from August through October [12]. In Tanzania, the peak season is from December through March.

GENETICS OF VIRULENCE

The genome of *Y. pestis* and its 3 plasmids was published in 2001 [21]. Molecular clock analysis suggests that *Y. pestis* emerged as a clone of *Yersinia pseudotuberculosis* ~20,000 years ago by acquiring 2 virulence-associated plasmids that carried genes enabling fleabite transmission and by silencing genes that

facilitated enteric transmission [22]. All 3 species of pathogenic *Yersinia*—*Y. pestis*, *Yersinia enterocolitica*, and *Y. pseudotuberculosis*—carry 1 of the virulence plasmids with ~70 kilobases (kb) of DNA called the low-calcium response plasmid, or pYV or pCD1, because it encodes for a type III secretion system and yersinial outer proteins (Yops) and V antigen, which are expressed when bacterial growth is restricted by low concentrations of calcium at 37°C (Table 2). Only *Y. pestis* has an additional ~100-kb plasmid called pFra or pMT1, which encodes an antiphagocytic capsular protein called fraction 1 (F1) antigen and the murine toxin, as well as 9.5-kb plasmid called pPst or pPCP1, which encodes the plasminogen activator and the bacteriocin pesticin. Death ensues after initial intracellular growth of bacteria in mononuclear phagocytes, followed by explosive extracellular proliferation of organisms, resulting in high-grade bacteremia along with inflammation and necrosis in lymph nodes, spleen, and liver [37, 38]. Other virulence factors that were recently proposed include outer membrane lipoprotein [39] and adhesins that allow bacteria to adhere to epithelial cells [40].

Although plague evokes fatal inflammatory disease in animals and humans, recent work has elucidated important anti-inflammatory mechanisms for pathogenesis and maintenance of infection in nature. Perpetuation of plague through fleabite

Table 3. Diagnostic Methods for Detection of *Yersinia pestis* in Clinical Specimens

Method	Sensitivity, specificity, and rapidity
Culture of bubo aspirate, blood, or sputum specimen	Highly sensitive if patient is untreated, highly specific, takes 2–3 days for identification
Gram or Wayson stain of bubo aspirate or sputum specimen	Moderately sensitive, moderately specific, rapid within minutes
Immunofluorescent antibody applied to bubo aspirate or sputum specimen	Moderately sensitive, highly specific, rapid within minutes [45]
ELISA for F1 antigen in bubo aspirate	Highly sensitive, highly specific, rapid within hours [46]
Dipstick for F1 antigen in bubo aspirate	Highly sensitive, highly specific, rapid within minutes [47]
PCR for F1 gene in bubo aspirate	Moderately sensitive, highly specific, rapid within hours [48]

NOTE. ELISA, enzyme-linked immunosorbent assay; F1, fraction 1; PCR, polymerase chain reaction.

transmission requires that *Y. pestis* attain extraordinary concentrations in rodent blood ($\sim 10^8$ bacteria/mL) for fleas, which ingest $\sim 0.1 \mu\text{L}$ and need $\sim 10^4$ bacteria for an infective dose, to transmit infection to their next hosts [41]. This has been achieved during evolution by keeping rodents alive in the face of high-grade terminal bacteremia through the anti-inflammatory power of V antigen [42], less toxic lipopolysaccharide [43], reduced immunogenicity of lipopolysaccharide by elimination of O groups [44], accumulation of extracellular Yops, and the ability of F1 to block phagocytosis. *Y. pestis* is effective at multiplying rapidly in host tissues, largely because of its evasion of innate immune functions. In addition to elaboration of Yops and V antigen, which suppress cytokine production and function of phagocytes, the lipid A of lipopolysaccharide switches from hexa-acylated molecules produced at a temperature of 26°C, typically found in fleas, to tetra-acylated molecules produced at 37°C, found in mammals. Although the hexa-acylated lipid A is recognized by Toll-like receptor 4 and leads to proinflammatory cytokine elaboration, the tetra-acylated lipid A is nonstimulatory for Toll-like receptor 4, actually preventing activation of macrophages and antagonizing secretion of proinflammatory cytokines and activation of dendritic cells required for adaptive immunity [36].

LABORATORY DIAGNOSIS

The mainstay of rapid, bedside diagnosis of bubonic plague is examination of the bubo aspirate. A sterile needle on a syringe containing 1 mL of sterile saline is inserted through the skin into the center of the bubo. Saline is injected and immediately aspirated by vigorous withdrawal of the plunger, until blood-tinged liquid appears in the syringe. A drop is placed onto a microscope slide for Gram stain or Wayson stain, which contains methylene blue. A diagnostic specimen contains many gram-negative bacilli or blue bacilli after Wayson stain. Since the discovery of the causative bacterium by Alexandre Yersin in Hong Kong in 1894, isolation of the organism by culture has been the traditional diagnostic method of choice. It is also the reference standard for a diagnosis of plague, but newer methods of immunodiagnosis and PCR have been developed (Table 3). Patients with F1 antigen in the blood showed a mortality rate of 17% in Madagascar, and most of those who died had concentrations of antigen $>1 \mu\text{g/mL}$ [49]. A rapid dipstick test for antigen that uses 2 monoclonal antibodies against F1 antigen (one as a capture antibody on a nitrocellulose strip and the other attached to colloidal gold particles on a polyester release pad) has been developed for field use [47]. The Pasteur Institute workers applied the dipstick to bubo

Table 4. Antimicrobial Agents for Treatment of Plague

Drug	Clinical experience
Gentamicin	Monotherapy shown to be effective in the past decade in the United States [62] and Tanzania [63] after streptomycin therapy was discontinued
Streptomycin	Drug of choice from 1948 to ~ 40 years later, when its use was discontinued in most countries; still used in Madagascar in combination with trimethoprim-sulfamethoxazole
Doxycycline or tetracycline	Effective alternative to gentamicin when oral therapy is preferred; used as prophylaxis for pneumonic disease
Trimethoprim-sulfamethoxazole	Used in combination with streptomycin in Madagascar; recommended as prophylaxis for pneumonic disease [20]
Chloramphenicol	Effective but rarely used because of bone marrow toxic effects
Cephalosporins and other β -lactams	Not recommended but effective in experimental animal infection [56]
Ciprofloxacin and other fluoroquinolones	Not recommended but effective in experimental animal infection [57]

aspirates and other specimens in Madagascar, finding it highly specific and potentially more sensitive than both culture and F1 enzyme-linked immunosorbent assay. In an effort to modernize plague diagnosis, they propose using the dipstick at the bedside, omitting the cumbersome microscopic examination that is subject to vagaries of human judgment, and confirming diagnosis by culture. Hemagglutinating antibodies against F1 antigen increase in serum during the first week of illness and can be measured for evidence of plague infection. Paired serum samples, from the acute and convalescent phases of illness, should demonstrate a ≥ 4 -fold increase in antibody titer; a single serum specimen with a titer $\geq 1:10$ is diagnostic. An enzyme-linked immunosorbent assay is available to measure antibodies against F1 antigen in both classes of immunoglobulin M, signifying recent or current infection, and immunoglobulin G, signifying infection at a more remote time. PCR tests that use structural genes for F1 antigen, plasminogen activator, and murine toxin have been developed and may prove useful as specific and rapid tests for plague diagnosis. In sputum specimens to which *Y. pestis* was added, a real-time PCR probe for F1 antigen in extracted DNA detected 10^2 – 10^4 colony-forming units/mL in <5 h, with sensitivity dependent on inhibitors of the reaction in sputum [50]. Another real-time PCR with probes for 16S ribosomal RNA and the 3 plague virulence factors was highly sensitive and specific, with results in 3 h [51]. A need for these rapid genetic tests has been driven by preparations for bioterrorism defense, because they can be adapted for use in the field by persons without microbiological training.

ANTIMICROBIAL THERAPY

In vitro. *Y. pestis* isolates from human cases have generally shown consistent susceptibility to β -lactams, tetracyclines, aminoglycosides, chloramphenicol, and fluoroquinolones [52–54]. On the other hand, most isolates were resistant to colistin, polymyxin B, and macrolides, including clarithromycin [52]. Only 2 human isolates, both from Madagascar in 1995, have been reported to be highly resistant to drugs currently used to treat plague. The first case of drug resistance occurred in a 16-year-old boy with bubonic plague. The organism was resistant to streptomycin, chloramphenicol, tetracycline, sulfonamide, ampicillin, kanamycin, and spectinomycin. It was susceptible to trimethoprim, which was the drug credited with saving his life, because he had received treatment with streptomycin plus the combination of trimethoprim and sulfamethoxazole. This multidrug resistance was carried by a 150-kb plasmid, which was transferable in vitro to *Escherichia coli* and back to *Y. pestis*. The second case occurred in a 14-year-old boy who was infected with a strain that was resistant only to streptomycin. He also recovered while receiving treatment with trimethoprim-sulfamethoxazole. This resistance was encoded by a 40-kb plasmid

that was transferable to other isolates of *Y. pestis* and was demonstrated to be transferable to other species in the flea gut [55].

In vivo. When mice have been challenged with virulent *Y. pestis* by the subcutaneous, intravenous, or intranasal route, fatal systemic disease results that simulates human bubonic and pneumonic plague. For intravenous or subcutaneous infection, treatment with streptomycin, ceftriaxone, doxycycline, ciprofloxacin, and ofloxacin was effective in prevention of death [56, 57]. For intranasal infections, treatment with streptomycin, ciprofloxacin, and ofloxacin was most effective [58]. Cephalosporins, including ceftriaxone, were effective when administered 24 h after challenge but were inferior to aminoglycosides and fluoroquinolones when administered 42 h after challenge [58]. The third-generation fluoroquinolones, gatifloxacin and moxifloxacin, were effective in both systemic and pneumonic models of infection [59]. In mice infected intranasally and made neutropenic by cyclophosphamide, both levofloxacin and gentamicin, which are bactericidal drugs, were highly effective. Doxycycline was effective in nonneutropenic mice, but this bacteriostatic antibiotic allowed regrowth of residual bacteria in neutropenic mice [60]. A further advantage of fluoroquinolones is that, when drug-resistant mutants of *Y. pestis* were tested for pathogenicity in a neutropenic mouse thigh model, levofloxacin-resistant mutants were less pathogenic than were streptomycin-resistant mutants [61]. Human infections have been successfully treated for 60 years with several drugs used alone or in combination with others (Table 4). The drug of choice in Madagascar remains streptomycin, but in other countries where streptomycin is not available, gentamicin is an effective substitute [62]. A randomized comparison of gentamicin and doxycycline in Tanzania, however, indicated that doxycycline was equally effective, without any nephrotoxicity [63]. Thus, doxycycline can be considered an alternative drug of choice. Only 1 patient was reported to have received successful treatment with ciprofloxacin [64], but neither the fluoroquinolones nor the β -lactams have been subjected to testing in humans.

BIOTERRORISM AND VACCINES

Unlike anthrax, *Y. pestis* does not form spores and does not survive well outside the bodies of persons or animals. For this reason, no one has succeeded in developing an effective bio-weapon using aerosolized bacteria. In addition, the ability of pneumonic plague to propagate an epidemic is severely restricted by the requirement for close contact with a dying patient, usually on the last day of the patient's life [20]. Thus, the danger of terrorists using this organism has been greatly exaggerated [65–67]. Nevertheless, efforts are under way to develop new subunit vaccines that will protect persons against plague pneumonia. Earlier vaccines to prevent fleaborne plague have been used for more than half a century for persons in

areas of endemicity, including >1 million US military personnel deployed to Vietnam in the 1960s and 1970s, but the formalin-killed whole-cell plague vaccine, which did not protect persons against pneumonic disease, was discontinued by its US manufacturer in 1998. The live, attenuated vaccine EV76 that lacks *pgm* genes has been used for a long time in Europe and other countries but is not commercially available. New subunit vaccines that contain F1 antigen and V antigen of *Y. pestis* are being tested for safety and immunogenicity. Although this is an active area of current research, doubts have been raised about whether subunit vaccines that engender antibody responses will protect against pneumonic plague, which has an intracellular phase and may require a cell-mediated immune response for protection [68].

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